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Binding of Benzidine, *N*-Acetylbenzidine, *N, N'*-Diacetylbenzidine and Direct Blue 6 to Rat Liver DNA

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Studies were performed to assess covalent binding of [^{3}H]benzidine, [^{14}C]*N*-acetylbenzidine, [^{14}C]*N, N'*-diacetylbenzidine, and the benzidine-derived azo dye Direct Blue 6 to rat hepatic DNA. Following IP injection into male Sprague-Dawley rats, benzidine and *N*-acetylbenzidine bound to liver DNA to yield the same adduct: *N*-(deoxyguanosin-8-yl)-*N'*-acetylbenzidine. The isomeric *N*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine and the deacetylated adduct *N*-(deoxyguanosin-8-yl) benzidine were also synthesized, but neither of these adducts was detected *in vivo*. Injection of *N,N'*-diacetylbenzidine resulted in only barely detectable binding which was insufficient for adduct analysis. [^{3}H]Direct Blue 6 was administered to male Wistar rats either by IP injection or by gavage. In both instances, Direct Blue 6 bound covalently to liver DNA; however, binding occurred at a much higher level in the IP injected animals. With IP injected animals, high pressure liquid chromatographic analysis indicated that approximately 70% of the radioactivity was associated with *N*-(deoxyguanosin-8-yl) benzidine.

Introduction

Benzidine (BZ) has been shown to induce tumors at various sites in laboratory animals (*1, 2*). The major target organ in the rat and mouse is the liver, whereas tumors of the bladder are observed in dogs. BZ is also known to be responsible for the induction of bladder cancer in humans occupationally exposed to this compound (*3, 4*). Morton et al. (*5*) have shown *in vitro* that BZ can be sequentially acetylated to *N*-acetylbenzidine (ABZ) and *N,N'*-diacetylbenzidine (DABZ). This latter species was then demonstrated to be a substrate for hepatic *N,O*-acyltransferase (*5, 6*) and hepatic sulfotransferase (*7*) and to form reactive intermediates that would covalently bind to nucleophiles. More recently, Morton and co-workers (*8*) reported that BZ and *N*-hydroxy-DABZ were more carcinogenic than DABZ in rats; however, the metabolic activation pathways responsible for tumor induction were not elucidated.

The BZ-derived azo dye, Direct Blue 6 (DBU6), will induce liver tumors in rats after a relatively short exposure period (*9*). When this or other BZ-based dyes are administered to animals, including man, BZ and its acetylated derivatives are detectable as urinary metabolites (*10-13*). Therefore, it has been suggested that the carcinogenicity of these compounds is likely to result from the action of the parent amine upon its release by microbial reduction of the dye in the alimentary tract. No data are yet available, however, to indicate the precise mechanism by which such compounds induce tumors. The work reported here and described in greater detail elsewhere (*14*) was initiated to determine the nature of the BZ-DNA adducts found in rat liver following administration of [^{3}H]BZ. Preliminary studies have also been performed on the DNA binding characteristics of [^{3}H]DBU6 in rats treated by IP injection or by gavage. Eventually, we hope to characterize the DNA adduct(s) formed from DBU6 and, by comparison with BZ, elucidate the pathway(s) by which such azo dyes are activated *in vivo*.

Materials and Methods

Animals

Experiments involving BZ, ABZ, and DABZ were performed with male Sprague-Dawley rats (9-23 weeks old, 250-350 g) obtained from the NCTR

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breeding colony and fed a pelleted Basal Diet (No. 5755C, Ralston Purina, St. Louis MO) *ad libitum*. Experiments involving DBU6 were performed with males of a Wistar-derived strain purchased from Bantin and Kingman, Ltd., Hull, UK, and fed diet 41B (Oxoid Ltd., London, UK).

Chemicals

All chemicals used were of analytical reagent grade. BZ, [ring-^{14}C]BZ (14.1 mCi/mmole), [ring-^{14}C]ABZ (17.9 mCi/mmole) and [ring-^{14}C]DABZ (17.1 mCi/mmole) were obtained from Dr. William P. Duncan, Midwest Research Institute, Kansas City, MO. [Acetyl-^{3}H]ABZ (45 mCi/mmole), [2,2′-^{3}H]BZ (115 mCi/mmole), and [2,2′-^{3}H]*N*-hydroxy-*N*′-ABZ (24.7 mCi/mmole) were synthesized by Dr. R. Roth of the Midwest Research Institute according to published procedures (*14*).

Preparation of [Ring-^{3}H]BZ

[Ring-^{3}H]BZ was synthesized from [ring-^{3}H]aniline (150 mCi/mmole) which was obtained from Radiochemical Centre, Amersham, UK. [^{3}H]Aniline (15.53 mg) was dissolved in 2.5 mL glacial acetic acid, 26 mg nitrosobenzene was added, and the reaction mixture was stirred overnight at room temperature. After centrifugation to remove unreacted nitrosobenzene, the supernatant which contained [ring-^{3}H]azobenzene was added to 5 mL hexane. The precipitate of nitrosobenzene was collected by centrifugation, was washed with hexane, and the washings were added to the hexane/acetic acid mixture. The solution of azobenzene was extracted with water and evaporated under reduced pressure. The [ring-^{3}H]azobenzene was then reduced and rearranged (*15*) to yield [ring-^{3}H]BZ (128 mCi/mmole; yield: 61.8% of theoretical).

Preparation of [Ring-^{3}H]DBU6

[Ring-^{3}H]BZ (128 mCi/mmole, 6.67 mg) was dissolved in 3 mL water which contained 22 μL concentrated HCl. The solution was cooled in ice and 5 mg $NaNO_2$ (1 mg/mL in water) was added dropwise until there was a slight excess of oxidant as shown by starch iodide paper. The solution was adjusted to pH 9 with saturated sodium carbonate and then 23.14 mg 1-naphthol-8-amine-3,6-disulfonic acid (H-acid; ICI Stevenston, Ayreshire, UK) in sodium carbonate solution, pH 9, was added. After 10 min, the reaction mixture was extracted twice with dichloromethane to remove any unreacted [^{3}H]BZ and then freeze dried. The synthetic [^{3}H]DBU6 was coinjected with an authentic standard onto a Partisil-10 ODS-2 analytical column (Whatman, Maidstone, Kent, UK) and eluted with a gradient of 1% cetrimide, pH 3 (A): 1% cetrimide in *n*-propanol (B). The gradient conditions were 20% B over 10 min, rising to 100% B over the next 15 min. Visible absorptivity was monitored at 580 nm and half-minute fractions were collected for scintillation counting. DBU6 eluted after 22 min and its radiochemical purity was >98%.

Binding of BZ, ABZ, and DABZ to Rat Liver DNA *In Vivo*

One male rat was injected IP with [2,2′-^{3}H]BZ, two rats were injected IP with [ring-^{14}C]ABZ, two with [ring−^{14}C]DABZ and three with [acetyl-^{3}H]ABZ. In each case, the dose administered was 25 mg/kg. Animals were killed 24 hr later by decapitation, and their livers were removed. DNA was isolated and purified by chloroform:isoamyl alcohol:phenol extraction and hydroxylapatite chromatography (*14*, *16*). Dried DNA samples were dissolved at 2 mg/mL in 5 *mM* Bis-Tris-HCl buffer, pH 7.1, which contained 5 *mM* $MgCl_2$. Aliquots of this solution were used for colorimetric estimation of DNA (*17*) and for scintillation counting to determine total DNA binding. Samples were then enzymatically hydrolyzed and prepared for reversed-phase HPLC analysis by extraction of the adduct into *n*-butanol (*14*). Only one peak of radioactivity was eluted from the column following injection of samples from either BZ- or ABZ-treated animals. Fractions associated with this peak were pooled and used to determine the pH-dependent solvent partitioning profile of the adduct (*18*).

Reaction of *N*-Hydroxy-*N*′-ABZ With DNA

[2,2′-^{3}H]*N*-Hydroxy-*N*′-ABZ [2 mL of 25 *mM* solution in ethanol: DMSO (4:1)] was reacted with 500 mg calf thymus DNA in 100 mL argon-saturated 10 *mM* potassium citrate/1 *mM* EDTA buffer, pH 4.6. After 4 hr, the DNA was isolated (*19*), enzymatically hydrolyzed and prepared for HPLC analysis in the same manner as the *in vivo* samples. The major adduct peak was purified on a Waters C_{18}-μBondapak Semiprep column by eluting with 45% methanol at a flow rate of 4 ml/min. The adduct was characterized by NMR and mass spectroscopy (*14*). A pH-dependent solvent partitioning profile was obtained and compared with that of the adduct formed *in vivo* from BZ or ABZ. The synthetic adduct was also coinjected with the radiolabeled *in vivo* adduct onto an analytical reversed-phase column. The column was eluted at 2 mL/min with 20% methanol in water for 10 min followed by a linear gradient over 10 min to 70% ethanol, and then increasing to 100% methanol over 5 min. UV absorbance was monitored at 282 nm and half-minute fractions were collected for scintillation counting.

Carboxylesterase Treatment of Adducts

The purified synthetic adduct (1 mg) obtained as described above was dissolved in 100 μL DMSO and added to 10 mL 50 mM potassium phosphate buffer, pH 7.7. Carboxylesterase I and II (80 units each) were added and the mixture was incubated at 37°C for 4 hr. The single product was purified on preparative HPLC and was characterized by NMR and mass spectral analysis. The product of reaction between *N*-acetoxy-DABZ and deoxyguanosine was treated similarly and the final product was characterized by mass and NMR spectral measurements.

Binding of DBU6 to Rat Liver DNA

A total of 10 rats were treated with [ring-^{3}H]DBU6. Half of the animals were injected IP at a dose of 12 mg/kg, and the remaining rats were treated at the same dose by gavage. One rat from each group was killed at 2, 8, 24, 48, and 168 hr after treatment. Livers were removed and DNA was extracted and purified as described above. Aliquots of washed liver (100 mg) were dissolved in 2 mL of Soluene (Packard Instrument Co., Illinois, USA) and counted for radioactivity to determine total uptake of dye. Purified DNA was estimated colorimetrically and associated radioactivity determined by scintillation counting. Samples at 24 and 168 hr were enzymically hydrolyzed and analyzed on HPLC as described for BZ- and ABZ-modified DNA.

Results

Covalent Binding of BZ, ABZ and DABZ to Rat Liver DNA

[2,2′-^{3}H]BZ, [ring-^{14}C]ABZ and [acetyl-^{3}H]ABZ all bound covalently to rat liver following IP injection (Table 1). Enzymic hydrolysis and subsequent HPLC analysis of liver DNA from BZ- and ABZ-treated animals each resulted in detection of only one peak of radioactivity which coeluted at 19.5 min on an analytical HPLC reversed-phase column (Fig. 1). This suggested that the major DNA adduct induced in rat liver *in vivo* by BZ was identical to that induced by ABZ. The elution of a single peak of radioactivity at 19.5 min in [acetyl-^{3}H]ABZ-treated animals confirmed that the adduct was acetylated. When pH-dependent solvent partitioning experiments were performed on the purified DNA adduct from both BZ- or ABZ- treated animals, similar profiles were also observed (Fig. 2).

Table 1. Covalent binding of BZ, ABZ and DABZ to rat liver DNA.[a]

Treatment	pmole bound/mg DNA
[ring-^{3}H]BZ	48
[ring-^{3}H]ABZ	56-89
[acetyl-^{3}H]ABZ	61-72
[ring-^{3}H]DABZ	ND[b]

[a]Male rats were administered the compounds IP at 25 mg/kg and then killed after 24 hr. Hepatic DNA was isolated and quantified by the diphenylamine reaction, and the specific activity was determined by scintillation counting.

[b]ND = not detected. The limit of detection was <0.3 pmole bound/mg DNA.

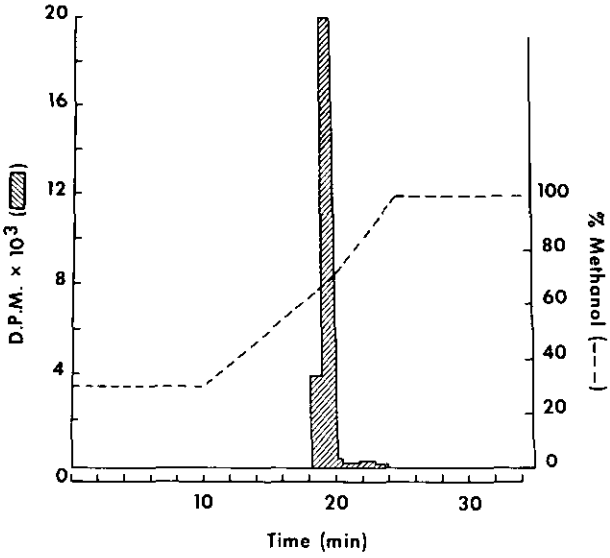

FIGURE 1. HPLC profile of liver DNA from BZ-treated rat. A male rat was inject IP with [2,2′-^{3}H]BZ and killed after 24 hr. Liver DNA was isolated, enzymatically hydrolyzed, and the adduct fraction was partitioned into *n*-butanol. Analysis was performed on a Whatman Partisil-10 ODS-2 column with the gradient indicated at a flow of 2 mL/min. The same profile was observed with [ring-^{14}C]ABZ and [acetyl-^{3}H]ABZ-treated rats.

Reaction of *N*-Hydroxy-*N*′-ABZ With DNA

[2,2′-^{3}H]*N*-Hydroxy-*N*′-ABZ was reacted with DNA at 37°C and the single adduct that formed was isolated after enzymic hydrolysis and extraction into *n*-butanol. Purification by reversed-phase HPLC yielded a product which was characterized by NMR and mass spectroscopy as *N*-(deoxyguanosin-8-yl)-*N*′-ABZ. The pH-dependent solvent partitioning profile (Fig. 2) and the chromatographic profile were identical to those obtained from the major *in vivo* adduct of either BZ or ABZ. This evidence, combined with pH stability and deacetylation data (*14*), confirmed the identity of the *in vivo* adduct as being *N*-(deoxyguanosin-8-yl)-*N*′-ABZ.

Synthesis of *N*-(Deoxyguanosin-8-yl)-*N*ABZ and *N*-(Deoxyguanosin-8-yl)-BZ

The isomeric acetylated derivative *N*-(deoxyguan-

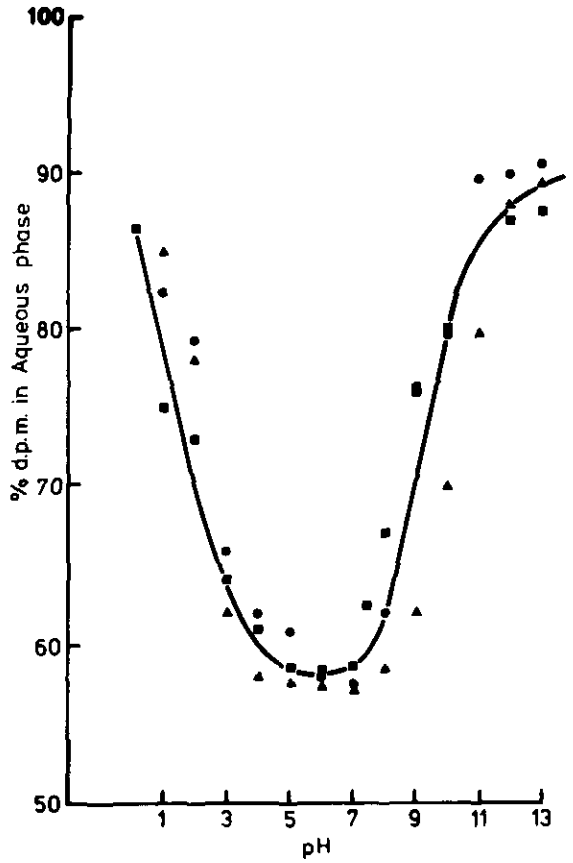

FIGURE 2. pH-dependent solvent partitioning profile of *N*-(deoxyguanosin-8-yl)-*N'*-ABZ (●) formed *in vivo* by injection of rats with BZ; (▲) from injection of rats with ABZ; (■) synthesized by reaction of *N*-hydroxy-*N'*-ABZ with deoxyguanosine.

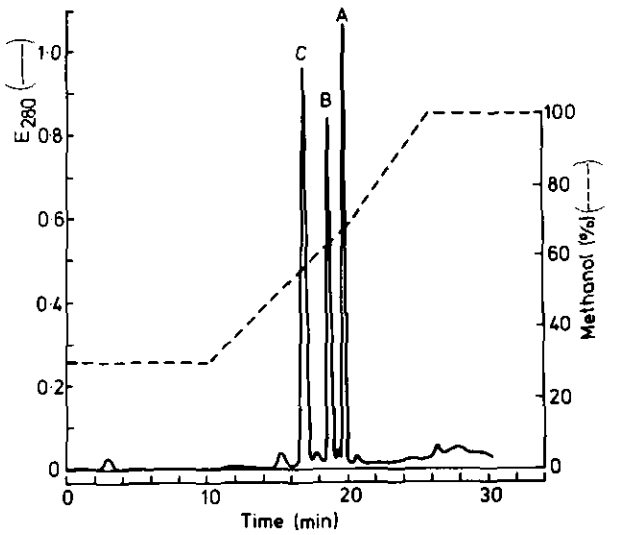

FIGURE 3. Coinjection onto a Partisil-10 ODS-2-reversed-phase HPLC column of (*A*) *N*-(deoxyguanosin-8-yl)-*N*-ABZ, (*B*) *N*-(deoxyguanosin-8-yl)-BZ and (*C*) *N*-(deoxyguanosin-8-yl-*N'*-ABZ.

osin-8-yl)-*N*-ABZ was synthesized by carboxylesterase-mediated, selective deacetylation of *N*-(deoxyguanosin-8-yl)-*N,N'*-DABZ. The deacetylated adduct *N*-(deoxyguanosin-8-yl)BZ was similarly synthesized from *N*-(deoxyguanosin-8-yl)-*N'*-ABZ. Both compounds were characterized by their mass and NMR spectra (*14*). The two acetylated and one deacetylated synthetic DNA adducts were coinjected onto an analytical reversed-phase HPLC column and all three compounds were well separated (*Fig. 3*). The *N*-butanol-extractable fraction of hydrolyzed liver DNA from rats treated with either BZ or ABZ was coinjected with these three marker compounds and radioactivity was found associated only with *N*-(deoxyguanosin-8-yl)-*N'*-ABZ.

Binding of DBU6 to Rat Liver DNA

Rats were administered [ring-^{3}H]-DBU6 either by gavage or by IP injection. Animals were killed at various times after treatment and total binding to liver tissue was measured (Table 2). Following extraction and purification, covalent binding to liver DNA was also measured (Table 2). In the case of orally dosed animals a small amount of DNA binding, which increased with time, was observed. A much larger extent of DNA binding was detected in IP injected animals. Samples from these latter rats were enzymically hydrolyzed and the adducts were analyzed by reversed-phase HPLC. Approximately 70% of the activity coeluted with *N*-(deoxyguanosin-8-yl)-BZ while the remaining 30% eluted later and has been tentatively identified as the deribosylated derivative.

Table 2. Total uptake of [^{3}H]DBU6 by rat liver and binding to DNA.

Time after treatment, hr	Administration route	Radioactivity, nCi/mg tissue[a]	Total binding, nmole/mole DNA (P)[b]
2	Oral	0.03	12
	IP	2.09	61
8	Oral	0.11	33
	IP	5.43	1589
24	Oral	0.12	64
	IP	5.46	1069
48	Oral	0.06	76
	IP	4.81	1384
168	Oral	0.06	111
	IP	7.16	7656

[a]Aliquots of washed liver (100 mg) were dissolved in 2 mL of Soluene and the extent of radioactivity was determined by liquid scintillation counting.

[b]Hepatic DNA was isolated and quantified by the diphenylamine reaction and the specific activity was determined by scintillation counting.

Discussion

In vitro studies by Morton et al. (*5*) have shown that *N,N'*-diacetylation of BZ occurred prior to *N*-hydroxylation. The hydroxamic acid produced by these two reactions could then be converted to a reactive species by cytosolic *N,O*-acyltransferase (*5,6*) or sulfotransferase (*7*). It was unclear, however, as to the pathway which led to the *in vivo* production of an electrophile capable of covalent reaction with cellular DNA. IP injection of either [ring-^{14}C]ABZ or [2,2'-^{3}H]BZ into rats resulted in the formation of the same DNA adduct. To determine whether acetylation of BZ or deacetylation of ABZ occurred, rats were injected with [acetyl-^{3}H]ABZ. The production of the same radiolabelled adduct confirmed that *N*-acetylation of BZ is the first step in its activation in rats. That *N*-hydroxylation of DABZ occurred to an appreciable extent *in vivo* seemed unlikely as injection of [ring-^{14}C]DABZ resulted in very low binding to DNA. To examine the possibility that *N*-hydroxylation may occur at the free amine of ABZ, *N*-hydroxy-*N'*-ABZ was synthesized and allowed to react with DNA at room temperature. Following hydrolysis of the DNA and chromatographic purification, the product was determined to be *N*-(deoxyguanosin-8-yl)-*N'*-ABZ. A variety of procedures, including cochromatography after incubation with base, acid, or carboxylesterase, and pH-dependent solvent partitioning, confirmed that the *in vivo* adduct and the synthetic marker were identical. The isomeric *N*-(deoxyguanosin-8-yl)-*N*-ABZ and the deacetylated adduct *N*-(deoxyguanosin-8-yl)-BZ were also synthesized and were well separated from the *in vivo* adduct by HPLC. Therefore, as the deacetylated adduct was not detected *in vivo*, it may be assumed that *N*-hydroxy-BZ or *N*-hydroxy-*N*-ABZ are not proximate carcinogenic species in hepatocarcinogenesis in rats. Furthermore, diacetylated adducts were not found, which demonstrates that if *N*-hydroxy-DABZ was formed during metabolism, it does not act as a substrate for sulfotransferase enzymes. Thus, *N*-hydroxy-*N'*-ABZ, whether formed by sequential *N*-acetylation and *N'*-hydroxylation of BZ or by deacetylation of *N*-hydroxy-DABZ, appears a strong candidate proximate carcinogenic species of BZ and ABZ. *N*-Hydroxy-*N'*-ABZ may, of course, be further activated by *O*-esterification or by intermolecular *N,O*-acyltransfer (*20*).

Direct Blue 6 is a polar dye derived from BZ by coupling it to two molecules of H-acid via diazo linkages. It has been suggested that the carcinogenicity of this and similar dyes results from reductive cleavage of the diazo bonds with consequent release of free BZ (*10, 21-23*). Such reduction can occur as a result of action by gut microflora (*22*) or possibly by mammalian liver enzymes (*24, 25*). Covalent binding of DBU6 to rat liver DNA occurred in animals dosed with [ring-^{3}H]DBU6 either by gavage or by IP injection. Total binding following IP injection was greater than that observed in orally treated animals. Total binding was greatest in the 7-day animals which could be explained by the slow release of the dye from tissues to which it is bound by ionic interaction. In contrast to the ABZ adduct obtained from BZ and ABZ treatments, the major adduct which resulted from DBU6 administration coeluted with *N*-(deoxyguanosin-8-yl)-BZ. It would appear from these preliminary data that metabolism of the azo dye is not as simple as reduction to BZ before activation to form a reactive intermediate.

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